

## Characterization and Genetic Complementation of a *Brucella abortus* High-Temperature-Requirement A (*htrA*) Deletion Mutant

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In order to evaluate the biological function of the *Brucella abortus* high-temperature-requirement A (HtrA) stress response protein homolog, the majority of the *htrA* gene was deleted from the chromosome of *B. abortus* 2308 via gene replacement. In contrast to the parental strain, the resulting *htrA* deletion mutant, designated PHE1, failed to grow on solid medium at 40°C and demonstrated increased sensitivity to killing by H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in disk sensitivity assays. BALB/c mice were infected with strains 2308 and PHE1 to assess the effect of the *htrA* mutation on virulence, and significantly fewer brucellae were recovered from the spleens of mice infected with PHE1 than from those of mice infected with 2308 at 1 week postinfection. Genetic complementation studies were performed to confirm the relationship between the *htrA* mutation and the phenotype observed for PHE1. Plasmid pRIE1 was constructed by inserting a 1.9-kb *Eco*RI fragment encoding the *B. abortus htrA* gene into the broad-host-range plasmid pBBR1MCS. Introduction of pRIE1 into PHE1 relieved the temperature- and H<sub>2</sub>O<sub>2</sub>-sensitive phenotypes of this mutant in vitro, and PHE1(pRIE1) colonized the spleens of BALB/c mice at levels equivalent to those of the parental 2308 strain at 1 week postinfection. These results support our previous proposal that the *B. abortus htrA* gene product functions as a stress response protein and further suggest that this protein contributes to virulence. These studies also demonstrate the utility of the broad-host-range plasmid pBBR1MCS for genetic complementation studies in *Brucella* spp., establishing a key reagent for more detailed genetic analysis of this important zoonotic pathogen.

*Brucella abortus* is a gram-negative zoonotic pathogen which causes abortion and infertility in cattle and a condition known as undulant fever in humans (20). Survival and replication in host phagocytes, particularly macrophages, are critical to the pathogenesis of *Brucella* infections. Cell-mediated immunity and the subsequent activation of macrophages are essential for host clearance of infection, and oxidative killing pathways are thought to be the primary mechanism by which host phagocytes eliminate intracellular brucellae (2, 5, 10). Oxidative damage targets cellular proteins for degradation, and the proteases which remove these damaged proteins before they accumulate to toxic levels represent an important component of a bacterial cell's defense against oxidative damage (7). Several biochemical studies have implicated the *Escherichia coli* high-temperature-requirement A (HtrA) protein (16), also known as DegP (28) and protease Do (26), in the degradation of oxidatively damaged proteins (8). This biochemical evidence is supported by the observation that *Salmonella typhimurium htrA* mutants show significant increases in sensitivity to oxidative killing in vitro in comparison to wild-type strains (13) and fail to replicate in cultured macrophages (3). *S. typhimurium htrA* mutants are also highly attenuated in BALB/c mice (6, 18), and it has been proposed that their inability to withstand oxidative killing within host phagocytes is the basis for their attenuation (13).

The gene encoding the immunoreactive *B. abortus* HtrA homolog has been cloned and characterized in our laboratory (22). On the basis of the predicted similarity of *B. abortus* HtrA

to previously described HtrA proteins, we proposed that the *B. abortus htrA* gene product functions as a cellular stress response protein. This report describes the creation and characterization of a *B. abortus htrA* deletion mutant and presents evidence supporting a biological function for the *B. abortus htrA* gene product similar to that which has been described for the *E. coli* (16, 28) and *S. typhimurium* (13) HtrA proteins. Results indicating that the *B. abortus htrA* gene product contributes to the virulence of *B. abortus* in the BALB/c mouse model are also presented.

### MATERIALS AND METHODS

**Construction of a *B. abortus htrA* deletion mutant.** The majority of the *htrA* gene was removed from the chromosome of virulent *B. abortus* 2308 by the gene replacement strategy originally described by Halling et al. (11). Plasmid pPS1 was constructed by removing two internal *Bss*HII fragments from the 1.9-kb *Eco*RI fragment encoding the *B. abortus htrA* gene in pBA323 (22) and replacing them with a 1.8-kb *Bam*HI-*Hind*III fragment containing the kanamycin resistance gene from transposon *TnphoA* (17). pPS1 was introduced into *B. abortus* 2308 by the procedures outlined by Lai et al. (15). Because pPS1 is a ColE1-based plasmid, it is incapable of autonomous replication in *Brucella* spp. (11, 21); thus, ampicillin- and/or kanamycin-resistant transformants result from integration of antibiotic resistance genes carried on the plasmid vector via homologous recombination into the *Brucella* chromosome (Fig. 1). Transformants were selected on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) and 45 µg of kanamycin per ml (SBak) at 37°C under an atmosphere containing 5% CO<sub>2</sub>. Kanamycin-resistant transformants were further evaluated for growth on SBak supplemented with 100 µg of ampicillin per ml (SBaka) to detect

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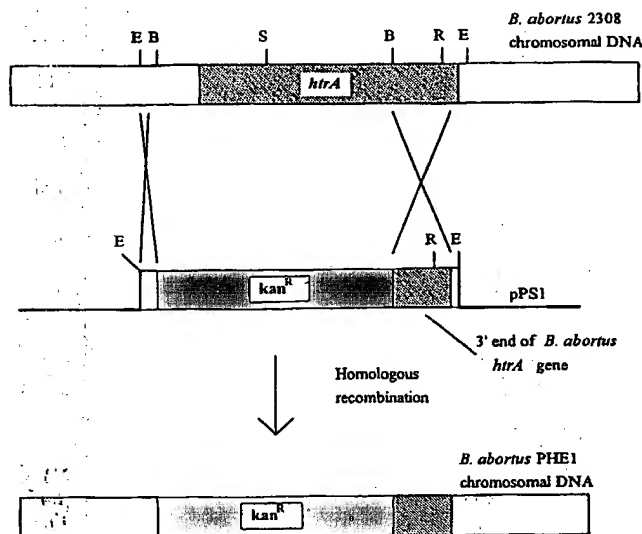


FIG. 1. Gene replacement strategy used for the creation of the *B. abortus htrA* mutant PHE1. The construction of pPS1 is described in the text. The open regions represent *B. abortus* chromosomal DNA, the hatched regions represent the *B. abortus htrA* gene, and the solid regions represent the kanamycin resistance cartridge from *TnphoA*. Abbreviations: E, *EcoRI*; B, *Bss*HII; S, *Sma*I; R, *EcoRV*.

transformants resulting from single-crossover insertion of the kanamycin resistance gene into the 2308 chromosome.

To confirm gene replacement in the putative *B. abortus htrA* mutants and the failure of these mutants to produce the HtrA protein, genomic DNA and whole-cell lysates were prepared from the mutants and the parental 2308 strain, and Southern and Western (immunoblot) analyses were performed by previously described procedures (22, 23). An *htrA*-specific probe was prepared from an internal 500-bp *EcoRV* fragment obtained from the *htrA* coding region in pBA323 (22). A kanamycin cartridge-specific probe was prepared from the *Bam*HI-*Hind*III fragment containing the *TnphoA* kanamycin resistance gene used for gene replacement, and a vector-specific probe was constructed from pUC19 linearized with *Sall*. Probes were labeled with [ $\alpha$ - $^{32}$ P]dCTP by nick translation procedures (24). Western blot analysis was performed with a monoclonal antibody specific for the perosamine O-chain of the *B. abortus* lipopolysaccharide (BRU38) (25), serum from a goat hyperimmunized with *B. abortus* RB51 (23), and antiserum specific for *B. abortus* HtrA prepared in the following manner. A cloned 1.5-kb *Nae*I-*Eco*RI fragment encoding the C-terminal 476 amino acids of the *B. abortus* HtrA protein was inserted into pRSETC (Invitrogen, San Diego, Calif.), and the resulting fusion protein, consisting of the N-terminal 41 amino acids of the RSET peptide and amino acids 24 to 500 of the *B. abortus* HtrA protein, was overexpressed in *E. coli* JM109 by using a T7 polymerase-based expression system (29). Recombinant *E. coli* cells expressing the RSET-HtrA fusion protein were lysed by exposure to guanidinium HCl and brief sonication according to the directions supplied with the RSET system. The resulting cell lysate was dialyzed exhaustively against phosphate-buffered saline (PBS), and a portion (1 mg of protein) of this lysate was injected into a New Zealand White rabbit. Four weeks postimmunization, the rabbit was boosted by injection with 1 mg of the recombinant *E. coli* cell lysate, and 10 days following this booster immunization, blood was collected from the rabbit

after it had been killed by pentobarbital overdose and exsanguination, and serum was collected and stored at  $-20^{\circ}\text{C}$ . The Ribi adjuvant system (Ribi Immunochem, Hamilton, Mont.) was used for both primary and booster immunizations.

**Phenotypic characterization of the *B. abortus htrA* mutant.** Colony formation on duplicate SBA or SBak plates, one incubated at  $37^{\circ}\text{C}$  and the other at  $40^{\circ}\text{C}$ , was evaluated after 3 days of incubation with 5%  $\text{CO}_2$ . The sensitivity of *Brucella* strains to killing by  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  was evaluated by a modification of the disk sensitivity assay described by Johnson et al. (13). Strains were grown overnight in brucella broth, the cultures were adjusted to an optical density at 600 nm of 0.2, and 75  $\mu\text{l}$  of the bacterial suspensions was spread onto Schaedler agar plates. A 10-mm filter disk saturated with 10  $\mu\text{l}$  of a 30%  $\text{H}_2\text{O}_2$  solution (Sigma, St. Louis, Mo.) or 10  $\mu\text{l}$  of a 1-mg/ml solution of the redox cycling reagent menadione (Sigma) was placed in the center of each 85-mm diameter plate. Following growth for 72 h at  $37^{\circ}\text{C}$ , the diameter of the zone of clearance around each disk was measured.

**Experimental infection of BALB/c mice.** Bacterial strains were grown for 3 days on SBA or SBak, harvested in PBS, and adjusted to a standardized optical density approximating  $10^9$  CFU/ml, and female BALB/c mice (9 to 10 weeks old) were infected by intravenous injection of  $5 \times 10^4$  CFU in 0.1 ml of PBS (19). Infecting doses were confirmed by serial dilution and plating, and five mice were infected with each strain. One week after infection, the mice were exsanguinated via cardiac puncture, the spleens and livers were removed, and the numbers of brucellae per organ were determined by serial dilution and plating on SBA and expressed as log CFU per organ. Statistical comparisons between experimental groups were performed by the one-tailed Student's *t* test (27).

**Construction of pRIE1 and introduction of this plasmid into the *B. abortus htrA* mutant.** By previously described procedures (22, 23), a 1.9-kb *Eco*RI fragment containing the *htrA* gene and its putative promoter region from *B. abortus* 2308 was isolated from plasmid pBA323 and inserted into the *Eco*RI site residing in the multiple cloning site of pBBR1MCS (14). Plasmid pBBR1MCS and pBBR1MCS-based constructs were introduced into *B. abortus* strains by electroporation (15), and transformants were selected on SBak supplemented with 30  $\mu\text{g}$  of chloramphenicol per ml (SBakc) at  $37^{\circ}\text{C}$  under an atmosphere of 5%  $\text{CO}_2$ .

**Determination of extrachromosomal location and copy number of pRIE1 in *B. abortus*.** Total genomic DNA preparations (23) from PHE1(pRIE1) and PHE1(pBBR1MCS) were subjected to electrophoresis in 0.4% agarose gels to separate chromosomal DNA from plasmid DNA and transferred to nitrocellulose, and Southern blot analysis was performed with the *htrA*-specific probe and a 0.96-kb vector-specific probe containing the chloramphenicol resistance gene from pBBR1MCS. To determine the copy number of pRIE1 in *B. abortus*, Southern blot analysis was performed on total genomic DNA from *B. abortus* 2308 (which has one copy of the *htrA* gene present in the chromosome) and 2308(pRIE1) with the *htrA*-specific probe. Both undigested genomic DNA preparations separated on 0.4% agarose gels and genomic preparations digested with *Bam*HI and *Hind*III, which yields both a 1.9-kb *htrA* fragment representing the plasmid-borne copy of the *htrA* gene and a 5.7-kb fragment representing the chromosomal copy of the *htrA* gene, were examined in these experiments. The relative intensities of the hybridizing bands were compared by using the Molecular Dynamics model 425E PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

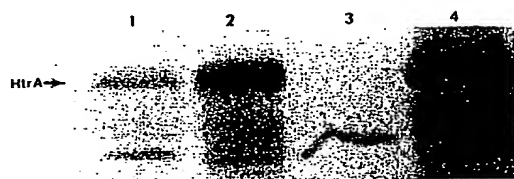


FIG. 2. Western blot analysis of rabbit anti-*Brucella* HtrA antiserum reacted with whole-cell extracts of *B. abortus* 2308 and PHE1. Lanes: 1, *B. abortus* 2308; 2, *B. abortus* 2308(pRIE1); 3, *B. abortus htrA* mutant PHE1; 4, *B. abortus* PHE1(pRIE1).

### RESULTS

Gene replacement was used to generate *B. abortus htrA* mutants. A kanamycin-resistant, ampicillin-sensitive transformant resulting from the introduction of the replacement vector pPS1 into *B. abortus* 2308 was selected for further study and given the designation PHE1. To confirm that the majority of the *htrA* gene had been deleted from the PHE1 chromosome as the result of the gene replacement event depicted in Fig. 1, Southern blot analysis was performed on chromosomal DNA prepared from strains PHE1 and 2308 (data not shown). DNA from a transformant designated PHE2, which was resistant to both kanamycin and ampicillin and suspected to be the result of a single-crossover event, was also examined in these experiments. As expected, the *htrA*-specific probe hybridized with a single 2.2-kb band representing the kanamycin resistance gene from *TrpA* (1.8 kb) linked to the residual 387 nucleotides of the *htrA* gene in the PHE1 DNA. The *htrA*-specific probe reacted with a single 1.9-kb band representing the wild-type *htrA* in the 2308 DNA and with 1.9- and 2.2-kb bands in DNA from PHE2. Single 2.2-kb bands reactive with the kanamycin resistance gene-specific probe were only detected in DNA from PHE1 and PHE2, and a 2.7-kb band reactive with the pUC19-specific probe was only detected in the DNA from PHE2.

The failure of PHE1 to express the HtrA protein was confirmed by Western blot analysis of whole-cell lysates with *B. abortus* HtrA-specific antiserum (Fig. 2). The identity of PHE1 was further confirmed by Western blot analysis with the *B. abortus*-specific hyperimmune goat serum, and reactions with BRU38 revealed identical lipopolysaccharide O-chain profiles for PHE1 and the parental 2308 strain in immunoblots (data not shown).

Initial characterization of PHE1 revealed that while the parental 2308 strain produced isolated colonies after 3 days of incubation on SBA at 40°C, PHE1 did not. However, both strains produced isolated colonies on this medium after 3 days at 30 and 37°C. Comparable growth rates were also observed for PHE1 and 2308 in brucella broth cultures incubated at 30 or 37°C. In experiments designed to compare the sensitivity of PHE1 and 2308 to oxidative killing, significantly larger zones of inhibition ( $P < 0.001$ ) were observed for PHE1 than for 2308 after exposure to  $H_2O_2$ -saturated disks on solid medium (Fig. 3). Larger zones of inhibition were also detected for PHE1 ( $35.5 \pm 2.7$  mm) versus 2308 ( $24.2 \pm 0.9$  mm,  $P < 0.001$ ) when these strains were evaluated for sensitivity to the  $O_2^-$ -generating compound menadione. The results of the virulence studies in BALB/c mice demonstrated that mice infected with PHE1 showed significantly lower spleen counts ( $4.78 \pm 0.14$  log CFU per spleen) than mice infected with the parental 2308 strain ( $6.22 \pm 0.26$  log CFU per spleen) ( $P < 0.001$ ) at 1 week postinfection.

To confirm the relationship between the PHE1 phenotype

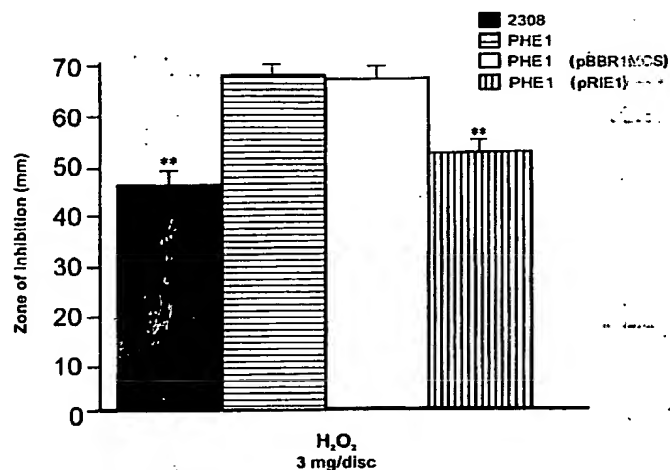


FIG. 3. Sensitivity of *B. abortus* strains 2308, PHE1, PHE1 (pBBR1MCS), and PHE1(pRIE1) to killing by  $H_2O_2$  on solid medium. The results presented represent the averages of five determinations, and the error bars indicate standard deviation. \*\*,  $P < 0.001$ .

and the *htrA* mutation, a recombinant plasmid was constructed by inserting the *B. abortus htrA* gene into the multiple cloning site of pBBR1MCS in an orientation opposite that of the direction of transcription directed from the *lacZ* promoter resident in this vector (Fig. 4). This plasmid was given the designation pRIE1. Introduction of pRIE1 into strain PHE1 restored the ability of the *B. abortus htrA* mutant to grow on solid medium at 40°C, while introduction of the parental

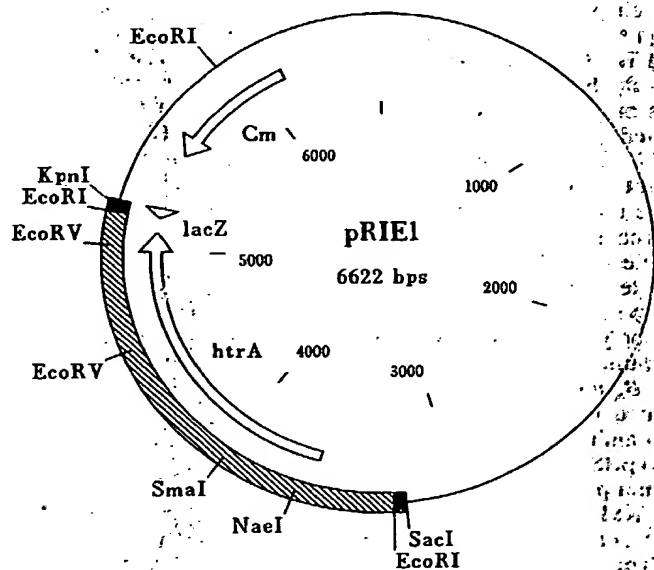


FIG. 4. Restriction map of pRIE1. The thin line represents pBBR1MCS sequences, and the thick, hatched line represents the cloned *B. abortus* DNA fragment containing the *htrA* gene, with the arrow denoting the direction of transcription of *htrA*. The multiple cloning site of pBBR1MCS is represented by thick black lines; the arrowhead labeled *lacZ* denotes the location of the *lacZ* promoter resident in pBBR1MCS and its direction of transcription; the arrow labeled *Cm* represents the chloramphenicol resistance gene in pBBR1MCS and its direction of transcription.

pBBR1MCS did not. Western blot analysis of *B. abortus* cell lysates confirmed a direct correlation between HtrA synthesis and the ability of strains to form colonies at 40°C (Fig. 2). PHE1(pRIE1) also demonstrated zones of inhibition in the H<sub>2</sub>O<sub>2</sub> killing assay that were significantly different from those obtained for PHE1 or PHE1(pBBR1MCS) and equivalent to those obtained for the parental 2308 strain (Fig. 3). Evaluation of the complemented mutant in the BALB/c mouse model revealed that the number of brucellae isolated from the spleens of mice infected with PHE1(pRIE1) at 1 week postinfection ( $6.14 \pm 0.18$  log CFU per spleen) was not significantly different from the levels obtained from mice infected with 2308 ( $6.22 \pm 0.26$  log CFU per spleen) ( $P < 0.05$ ) but was significantly higher than that obtained from mice infected with PHE1 ( $4.78 \pm 0.14$  log CFU per spleen) ( $P < 0.001$ ).

Digestion of plasmid DNA isolated from PHE1(pRIE1) with *Hind*III generated a single 6.6-kb band on agarose gel electrophoresis, consistent with the predicted size of pRIE1, and the presence of the *htrA* gene in pRIE1 was confirmed by Southern blot analysis with the 1.9-kb *Eco*RI fragment encoding the *B. abortus* *htrA* gene (data not shown). Southern blot analysis of total genomic DNA preparations from PHE1(pRIE1) and PHE1(pBBR1MCS) demonstrated that the *htrA*- and vector-specific probes reacted only with the plasmid DNA, confirming that pRIE1 and pBBR1MCS had not integrated into the PHE1 chromosome. Separate Southern blot analyses in which quantitative autoradiography was used to compare the ratio of plasmid-specific signals to chromosome-specific signals indicated that pRIE1 is maintained at approximately 10 copies per cell in *B. abortus* 2308 (data not shown).

## DISCUSSION

Members of the high-temperature-requirement A (HtrA) class of stress response proteins are serine proteases which apparently function by degrading oxidatively or otherwise damaged proteins before they can accumulate to toxic levels in cells (7, 8, 13, 16, 28). Biochemical and genetic evidence indicates that these proteins represent important components of the cell's defenses against oxidative damage. For example, the HtrA protein (also known as protease Do) is one of several *E. coli* proteases which actively degrade oxidized peptides in vitro (7, 8), and *S. typhimurium* *htrA* mutants show a significant increase in sensitivity to killing by H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in vitro compared with parental strains (13). In *E. coli*, the HtrA protein is also required for growth at elevated temperatures (16), and this property was the basis for its initial identification and its present designation. In view of the characteristic properties of previously described HtrA proteins, the temperature-, H<sub>2</sub>O<sub>2</sub>-, and O<sub>2</sub><sup>-</sup>-sensitive phenotypes of the *B. abortus* mutant PHE1 described in this report are consistent with our previous proposal (22) that the *B. abortus* *htrA* gene product functions as a stress response protein.

It has been postulated that bacterial stress response proteins play important roles in allowing facultative intracellular pathogens to successfully adapt to the harsh environment of the host phagosome (1, 4), and experimental evidence derived from studies of *S. typhimurium* *htrA* mutants in cultured macrophages supports this hypothesis (3). Oxidative killing pathways are generally thought to be the primary mechanism by which host phagocytes kill intracellular brucellae (2, 5, 10, 12); thus, the HtrA protein may contribute significantly to successful survival in host phagosomes. In this regard, the attenuation of the *B. abortus* *htrA* mutant observed in BALB/c mice at 1 week postinfection may be the result of a defect in the ability of PHE1 to withstand oxidative killing in host phagocytes com-

pared with the parental 2308 strain. To more precisely determine the basis for the attenuation of PHE1, we plan to compare the survival and replication of PHE1 with that of the parental 2308 strain in murine neutrophils and macrophages in culture. Metabolic inhibitors and quenchers of specific reactive oxygen intermediates (12) will be used in these studies to confirm the role of oxidative killing in any differences in the survival of these strains in cultured phagocytes. Experiments are also under way to evaluate the effects of the *htrA* mutation on the long-term (20 weeks) colonization profile of *B. abortus* in BALB/c mice.

The positive correlation between the loss of the *B. abortus* *htrA* gene product and the phenotype exhibited by PHE1 in this study was initially established by Western blot analysis of whole-cell lysates with HtrA-specific antiserum and Southern blot analysis with *htrA*-specific probes. Confirmation of this relationship was obtained by introduction of a cloned copy of the *htrA* gene into PHE1 on the broad-host-range vector pBBR1MCS (14), which resulted in the return of this mutant to an essentially wild-type phenotype with respect to temperature and H<sub>2</sub>O<sub>2</sub> sensitivity and virulence in the mouse model. In addition to establishing the relationship between genotype and phenotype in PHE1, successful complementation with the cloned *htrA* gene ruled out the possibility that the PHE1 phenotype resulted from a polar effect on genes downstream from *htrA*. These results are important not only for confirming the genotype-phenotype relationship for PHE1, but also because they demonstrate the utility of pBBR1MCS for gene complementation in *Brucella* spp. Naturally occurring plasmids have not been reported for this genus, although plasmids of the IncP and IncW incompatibility groups have been shown to replicate in these organisms (21, 30). Indeed, the lack of reasonably small, well-characterized, and easily manipulated cloning vectors has hindered the genetic analysis of *Brucella* spp., especially with respect to the complementation of mutants. In this regard, pBBR1MCS offers several advantages, as evidenced by the present and related studies. The low copy number (10 copies per genome) makes this vector useful for genes encoding regulatory proteins or proteins which might be toxic at high levels. Preliminary studies with isolates obtained from the spleens of BALB/c mice on nonselective medium also suggest that pBBR1MCS-based derivatives are stably maintained for at least 4 weeks in the absence of selective pressure in *B. abortus* (9). Thus, it appears that pBBR1MCS should be a useful tool for genetic studies in this organism.

Because of the increased sensitivity of the *B. abortus* *htrA* mutant to oxidative killing and the attenuation of this mutant in the BALB/c mouse model, studies with the *htrA* mutation should enhance our understanding of the mechanisms by which host phagocytes are eventually able to kill intracellular brucellae. These types of studies will contribute significantly to our knowledge of the host-parasite interactions which occur during *Brucella* infections. *B. abortus* strains carrying the *htrA* mutation alone or in combination with other appropriate mutations may also be useful for the design of novel live vaccine candidates.

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